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PTO/SB/05 (2/98)

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for nonprovisional applications under 37 CFR § 1 53(b))

Attorney Docket No.	**481112.410**
First Inventor or Application Identifier	**James B. Dale**
Title	**GROUP A STREPTOCOCCAL VACCINES**
Express Mail Label No.	**EM001236728US**

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

1. ☐ General Authorization Form & Fee Transmittal
(Submit an original and a duplicate for fee processing)

2. **X** Specification [Total Pages] **38**
(preferred arrangement set forth below)
- Descriptive Title of the Invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings *(if filed)*
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3. **X** Drawing(s) *(35 USC 113)* [Total Sheets] **6**

4. Oath or Declaration [Total Pages] _____
   a. ☐ Newly executed (original or copy)
   b. ☐ Copy from a prior application (37 CFR 1.63(d))
   (for continuation/divisional with Box 17 completed)
   i. ☐ **DELETION OF INVENTOR(S)**
   Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)

5. Incorporation By Reference *(useable if box 4b is checked)*
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference therein. ☐

6. ☐ Microfiche Computer Program *(Appendix)*

7. Nucleotide and Amino Acid Sequence Submission *(if applicable, all necessary)*
   a. ☐ Computer-Readable Copy
   b. **X** Paper Copy (identical to computer copy)
   c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))

9. ☐ 37 CFR 3.73(b) Statement *(when there is an assignee)*   ☐ Power of Attorney

10. ☐ English Translation Document *(if applicable)*

11. ☐ Information Disclosure Statement (IDS)/PTO-1449   ☐ Copies of IDS Citations

12. ☐ Preliminary Amendment

13. **X** Return Receipt Postcard

14. ☐ Small Entity Statement(s)   ☐ Statement filed in prior application, Status still proper and desired

15. ☐ Certified Copy of Priority Document(s) *(if foreign priority is claimed)*

16. **X** Other: _Certificate of Express Mail_
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17. **If a CONTINUING APPLICATION,** *check appropriate box and supply the requisite information below and in a preliminary amendment*

☐ Continuation   ☐ Divisional   ☐ Continuation-In-Part (CIP)   of prior Application No.: _____

Prior application information: Examiner _____   *Group / Art Unit* _____

X Claims the benefit of Provisional Application No. **60/058,635, filed 9/12/97** _____

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Applicant	:	James B. Dale, Memphis, Tennessee
Filed	:	September 10, 1998
For	:	GROUP A STREPTOCOCCAL VACCINES

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Jeanette West / Brunetta Ishman / Kennethia Ishman

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Enclosures:
   Postcard
   Form PTO/SB/05
   Specification, Sequence Listing, Claims, Abstract (38 pages)
   6 Sheets of Drawings (Figures 1-7B)

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GROUP A STREPTOCOCCAL VACCINES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of United States Provisional Application No. 60/058,635, filed September 12, 1997, which application is incorporated by reference in its entirety.

TECHNICAL FIELD

The present invention provides pharmaceutical compositions and methods, and in particular, vaccines for use in preventing Group A streptococcal infections.

BACKGROUND OF THE INVENTION

Streptococci are a group of bacteria with the capacity to grow in chains. Many varieties are part of the normal bacterial flora in humans and are not especially harmful. However, a particular group of streptococcal bacteria, called group A and represented by *Streptococcus pyogenes*, is a human pathogen. Briefly, group A streptococci cause a variety of human illnesses, ranging from uncomplicated pharyngitis and pyoderma to life-threatening infections associated with toxic shock syndrome, deep tissue invasion and sepsis. In some individuals, untreated streptococcal pharyngitis may be followed by acute rheumatic fever. In recent years there has been a dramatic increase in the incidence of severe streptococcal infections (Davies et al., "Invasive group A streptococcal infections in Ontario, Canada. Ontario group A Streptococcal study group," *N. Engl. J. Med. 335*:547-554, 1996) and in the incidence of rheumatic fever (Veasey et al., "Resurgence of acute rheumatic fever in the intermountain region of the United States," *N. Eng. J. Med. 316*:421-427, 1987).

Although streptococcal infections can be generally treated with antibiotics, in at least 4% of cases the infection leads to acute rheumatic fever. This

disease is particularly prevalent in developing countries such as India, where millions of school-age children are affected.

The present invention provides new Group A streptococcal vaccines with enhanced immunogenicity, and further, provides other related advantages.

5 SUMMARY OF THE INVENTION

Briefly stated, the present invention provides immunogenic synthetic fusion polypeptides which stimulate an immune response against Group A streptococci. Within one aspect such polypeptides comprise (a) at least two immunogenic polypetides from a Group A streptococci of at least 10 amino acids in

10 length which are capable of stimulating an immune response against Group A streptococci, and a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic portion. Within preferred embodiments, the C-terminal peptide is not required to stimulate an immune response against Group A streptococci and hence, may be an inconsequential non-

15 immunogenic peptide, or a reiterated immunogenic polypeptide. Within certain embodiments, the immunogenic polypeptide can be obtained from a wide variety of Group A streptococci (ranging from "1" to greater than "90"), including for example, Types 1, 1.1, 2, 3, 4, 5, 6, 11, 12, 13, 14, 18, 19, 22, 24, 28, 30, 48, 49, 52, 55 and 56.

Within other aspects of the present invention, vaccinating agents are

20 provided for promoting an immune response against Group A streptococci, comprising (a) at least two immunogenic polypetides from a Group A streptococci of at least 10 amino acids in length which are capable of stimulating a protective immune response against Group A streptococci, and (b) a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic

25 portion, wherein the C-terminal peptide is not required to stimulate an immune response against Group A streptococci. As above, the polypeptide may selected from a wide variety of Group A streptococci (ranging from "1" to greater than "90"), including for example, types 1.1, 2, 3, 4, 5, 6, 11, 12, 13, 14, 18, 19, 22, 24, 28, 30, 48, 49, 52, 55 and 56. Within certain further embodiments, the vaccinating agent

30 may further comprise an adjuvant, such as, for example, alum, Freund's adjuvant,

and/or an immunomodulatory cofactor (*e.g.*, IL-4, IL-10, γ-IFN, or IL-2, IL-12 or IL-15).

Also provided are methods for vaccinating a host against Group A streptococci infections, comprising administering a vaccinating agent as described above.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of the hexavalent vaccine indicating the length of each emm gene fragment and the restriction sites that were synthesized into the original PCR primers. Each of the emm gene fragments starts at the first codon that encodes the mature native protein except the emm3 fragment, which represents codons 21-70.

Figure 2 is a SDS-polyacrylamide gel electrophoresis of the purified hexavalent protein stained with coomassie blue. Computer-assisted image analysis of the stained protein bands indicated that the hexavalent protein (M.W. 45 kDa) accounted for 90.3% of the total protein in the sample.

Figures 3A and 3B are ELISA's of antisera from three rabbits immunized with the hexavalent vaccine in either alum or CFA. Titers are expressed as the inverse of the last dilution of serum that resulted in an O.D. of >0.1. The ELISA antigen was the purified hexavalent protein. Each symbol represents serum from one rabbit.

Figures 4A-4F are ELISA's of antisera from rabbits immunized with the hexavalent protein in alum. Titers were determined using the purified pepsin-extracted M proteins (pep M) from the respective serotypes of group A streptococci. Each symbol represents one of three immunized rabbits.

Figure 5 depicts *in vitro* opsonization assays of antisera from rabbits immunized with the hexavalent protein in alum. Rotation mixtures consisted of the test organism, 0.1 ml of immune serum, and 0.4 ml of nonimmune human blood. The mixture was rotated for 45 minutes and the percentage of PMNs that had

5 ingested or were associated with streptococci was estimated by microscopic counts of stained smears. In each assay, the preimmune serum resulted in <10% percent opsonization. Each different bar represents serum from one of the three immunized rabbits.

Figure 6 is a graph which depicts opsonization of different strains

10 within the same serotype of group A streptococci promoted by hexavalent rabbit antisera. Each symbol represents a strain of group A streptococci of the serotype indicated on the horizontal axis. Opsonization assays were performed as described below in the Examples.

Figure 7 is a sequence of the hexavalent M protein vaccine (SEQ ID

15 NO:15 and SEQ ID NO:16).

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used

20 hereinafter.

"Vaccinating Agent" refers to a composition which is capable of stimulating a protective immune response within the host which receives the vaccinating agent. The vaccinating agent may be either protein, or, DNA-based (*e.g.*, a gene delivery vehicle). Within further aspects, a prokaryotic host may be generated

25 to be a vaccinating agent, and designed to express an immunogenic polypeptide or multivalent construct of the present invention (*see*, *e.g.*, U.S. Application No. 07/540,586).

"Gene delivery vehicle" refers to a recombinant vehicle, such as a recombinant viral vector, a nucleic acid vector (such as plasmid), a naked nucleic

30 acid molecule such as genes, a nucleic acid molecule complexed to a polycationic

molecule capable of neutralizing the negative charge on the nucleic acid molecule and condensing the nucleic acid molecule into a compact molecule, a nucleic acid associated with a liposome (Wang et al., *PNAS 84*:7851, 1987), a bacterium, and certain eukaryotic cells such as a producer cell, that are capable of delivering a

5   nucleic acid molecule having one or more desirable properties to host cells in an organism.

As noted above, the present invention provides vaccinating agents suitable for preventing Group A streptococcal infections. Briefly, as described in more detail below it has been discovered that, in order to optimize the

10   immunogenicity of all aspects of a multivalent vaccine. Within one aspect of the invention, immunogenic synthetic fusion polypeptides which stimulate an immune response against Group A streptococci are provided. Such polypeptides generally comprise (a) at least two immunogenic polypetides from a Group A streptococci of at least 10 amino acids in length which are capable of stimulating an immune response

15   against Group A streptococci, and (b) a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic portion, wherein the C-terminal peptide is not required to stimulate an immune response against Group A streptococci. Particularly preferred protective peptides are generally at least ten amino acids in length, and may be 30 amino acids or longer.

20   <u>Identification of Immunogenic Polypeptides, for Use in Vaccinating Agents</u>

Immunogenic polypeptides suitable for use within the present invention may be readily identified and generated given the disclosure of the subject application (*see also* Dale and Beachey, *J. Exp. Med. 163*:1191-1202; 1986; Beachey et al., *Nature 292*:457-459, 1981; Dale et al., *J. Immunol. 151*:2188-2194; 1993; and

25   U.S. Patent Nos. 4,454,121; 4,521,334; 4,597,967; 4,705,684; 4,919,930; and 5,124,153). Particularly preferred polypeptides can be obtained within the 50 amino acid residues of the N-terminus of an M protein.

Serotypes of Group A streptococci can be readily obtained from clinical isolates, from university collections (*e.g.*, Rockefeller University Collection,

30   1230 York Avenue, New York, NY) or from depositories such as the American Type

Culture Collection (10801 University Boulevard, Manassas, Virginia). Furthermore, sequences for Group A streptococci serotypes are available from the Centers for Disease Control, Atlanta, Georgia.

A.   Identification of Opsonic Epitopes of M Proteins

5   To demonstrate directly that opsonic M protein epitopes could be separated from autoimmune epitopes, peptides are copied from various serotypes (*e.g.*, the amino-terminal 20-50 amino acids of M5 (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of the type 24 M antigen," *J. Exp. Med.*

10   *145*:1469-1483, 1977). SM5(1-20) failed to react with affinity purified pep M5 heart-reactive antibodies (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of the type 24 M antigen," *J. Exp. Med. 145*:1469-1483, 1977). Rabbits immunized with SM5(1-20) coupled to tetanus toxoid developed high titers

15   of antibodies against pep M5 that opsonized type 5 streptococci (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of the type 24 M antigen," *J. Exp. Med. 145*:1469-1483, 1977). Most importantly, none of the immune sera crossreacted with human myocardium.

20

B.   Tissue-Crossreactive Epitopes of M Proteins

M proteins evoke antibodies that crossreact with a variety of human tissues and antigens within those tissues (Baird et al., "Epitopes of group A streptococcal M protein shared with antigens of articular cartilage and synovium," *J.*

25   *Immunol. 146*:3132-3137, 1991; Bronze, M.S. and Dale, J.B., "Epitopes of streptococcal M proteins that evoke antibodies that cross-react with human brain," *J. Immunol. 151*:2820-2828., 1993; Dale, J.B. and Beachey E.H., "Protective antigenic determinant of streptococcal M protein shared with sarcolemmal membrane protein of human heart," *J. Exp. Med. 156*:1165-1176, 1982). In order to determine cross-

reactivity, a series of overlapping peptides is synthesized that copies a selected fragment (*e.g.*, M5), and used to either inhibit or evoke tissue-crossreactive antibodies. For example, the myosin-crossreactive antibodies evoked by pep M5 in rabbits were almost totally inhibited by peptide 84-116 of pep M5. This peptide

5 spans the region between the A and B repeats of M5 and includes the degenerate A6 repeat. Murine and human myosin-crossreactive antibodies reacted with an epitope in peptide 183-189, which is located in the region between the B and C repeats of the intact M5 molecule.

Additional sarcolemmal membrane crossreactive epitopes are

10 localized to peptide 164-197. Several epitopes of M5 that evoked antibodies that crossreacted with articular cartilage and synovium can also be found within the B repeats and the region spanning the A and B repeats of M5. The brain-crossreactive epitopes of M6 that were shared with other M proteins are localized to the B repeat region of the molecule.

15 Many of the tissue-crossreactive epitopes are shared among types 5, 6, 18 and 19 M proteins (Bronze, M.S. and Dale, J.B., "Epitopes of streptococcal M proteins that evoke antibodies that cross-react with human brain," *J. Immunol.* *151*:2820-2828., 1993). Primary structural data reveals that all of these M proteins contain similar sequences within their B repeats (Dale et al., "Recombinant

20 tetravalent group A streptococcal M protein vaccine," *J. Immunol.* *151*:2188-2194, 1993; Dale et al., "Recombinant, octavalent group A streptococcal M protein vaccine," *Vaccine* *14*:944-948, 1996; Dale et al., "Type-specific immunogenicity of a chemically synthesized peptide fragment of type 5 streptococcal M protein," *J. Exp. Med.* *158*:1727-1732, 1983), which is most likely the location of the shared heart- ,

25 brain-, and joint-crossreactive epitopes.

It should be emphasized that it is not necessary to localize the tissue-specific epitope, but rather, to first localize protective epitopes and ensure that they are not tissue-reactive.

Once a suitable immunogenic polypeptide for a selected serotype has

30 been identified, it may be, optionally, combined with immunogenic polypeptides from other serotypes, in order to construct a multivalent vaccine. In this regard,

preferred vaccines include vaccines developed from a combination of serotypes such as 1, 1.1, 2, 3, 4, 5, 6, 11, 12, 13, 14, 18, 19, 22, 24, 28, 30, 48, 49, 52 and 56 (for serotype 30 see Nakashima et al., *Clinic Infec. Dis. 25*:260, 1997). Representative examples include vaccine such as 24, 5, 6, 19, 1, 3, X; and 1, 3, 5, 6, 18, 19, 22, 24,

5   28, 30, and X, wherein X is the C-terminal protective polypeptide.

PREPARATION OF VACCINATING AGENTS

Vaccinating agents of the present invention can be synthesized chemically (see, *e.g.*, Beachey et al., *Nature 292*:457-459, 1981), or generated

10   recombinantly. For recombinant production, PCR primers can be synthesized to amplify desired 5'sequences of each emm gene, and each primer is extended to contain a unique restriction enzyme site used to ligate the individual PCR products in tandem.

As noted above, the C-terminal portion of the vaccinating agent is

15   constructed so as to contain a selective portion that can be lost or cleaved *in vivo* without affecting the efficacy of the vaccine. This may be accomplished by, for example, including an inconsequential non-immunogenic polypeptide at the end, or, including an immunogenic polypeptide that does not adversely impact the efficiency of the vaccine (*e.g.*, a reiterated immunogenic polypeptide may be included at the end

20   of the vaccine). Furthermore, protective antigens from unrelated pathogens can also be combined into a single polypeptide, which may circumvent the need for carriers. Vaccines against some pathogens might include T and B cell epitopes originally derived from different proteins on the same hybrid construct. Additionally, multivalent hybrid proteins may be sufficient conjugates in carbohydrate vaccines,

25   such as those for *S. pneumoniae*, *H. influenza* B or group B streptococci.

For protein expression, the multivalent genes are ligated into any suitable replicating plasmid which is used to transform an appropriate prokaryote host strain. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Suitable prokaryotic hosts cells for transformation include,

30   for example, *E. coli, Bacillus subtilis, Salmonella typhimurium*, and various other species within the genera *Pseudomonas, Streptomyces,* and *Staphylococcus*.

Expression vectors transfected into prokaryotic host cells generally comprise one or more phenotypic selectable markers such as, for example, a gene encoding proteins that confer antibiotic resistance or that supplies an autotrophic requirement, and an origin of replication recognized by the host to ensure

5   amplification within the host. Other useful expression vectors for prokaryotic host cells include a selectable marker of bacterial origin derived from commercially available plasmids. This selectable marker can comprise genetic elements of the cloning vector pBR322 (ATCC 37017). Briefly, pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying

10   transformed cells. The pBR322 "backbone" sections are combined with an appropriate promoter and a mammalian ETF structural gene sequence. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden), pQE30 and pGEM1 (Promega Biotec, Madison, WI, USA).

15   Common promoter sequences for use within prokaryotic expression vectors include β-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EPA 36,776) and tac promoter (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold

20   Spring Harbor Laboratory, (1989)). A particularly useful prokaryotic host cell expression system employs a phage λ P_L promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection that incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9 (ATCC 37092)) and pPLc28 (resident in *E. coli* RR1

25   (ATCC 53082)).

Transformation of the host strains of *E. coli* is accomplished by electroporation using standard methods (Dale et al., "Recombinant tetravalent group A streptococcal M protein vaccine," *J. Immunol.* 151:2188-2194, 1993; Dale et al., "Recombinant, octavalent group A streptococcal M protein vaccine," *Vaccine*

30   14:944-948, 1996). Successful transformants are identified by colony blots using

rabbit antisera raised against one of the native M proteins or a synthetic peptide copy of the amino-terminus of one of the M proteins included in the multivalent protein.

The molecular size and antigenicity of the recombinant protein expressed by selected clones are determined by performing Western blots of extracts

5   of *E. coli* (Dale et al., "Recombinant tetravalent group A streptococcal M protein vaccine," *J. Immunol. 151*:2188-2194, 1993) using rabbit antisera raised against each native M protein purified from pepsin extracts of live streptococci (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of the type 24 M antigen," *J.*

10   *Exp. Med. 145*:1469-1483, 1977). The multivalent gene is sequenced by the dideoxy-nucleotide chain termination method to confirm that each gene fragment is an exact copy of the native emm sequence.

GENE-DELIVERY VEHICLE-BASED VACCINES

15   Injection of mammals with gene delivery vehicles (*e.g.*, naked DNA) encoding antigens of various pathogens has been shown to result in protective immune responses (Ulmer et al., *Science 259*:1745-9, 1993; Bourne et al., *J Infect. Dis. 173*:800-7, 1996; Hoffman et al., *Vaccine 12*:1529-33, 1994). Since the original description of *in vivo* expression of foreign proteins from naked DNA injected into

20   muscle tissue (Wolff et al., *Science 247*:1465-8, 1990), there have been several advances in the design and delivery of DNA for purposes of vaccination.

The M protein vaccines described above are ideally suited for delivery via naked DNA because protective immunity is ultimately determined by antibodies. For example, within one embodiment the multivalent genes are ligated into plasmids

25   that are specifically engineered for mammalian cell expression (*see, e.g.*, Hartikka et al., *Hum Gene Ther 7*:1205-17, 1996, which contains the promoter/enhancer element from cytomegalovirus early gene, the signal peptide from human tissue plasminogen activator and a terminator element from the bovine growth hormone gene). The M protein hybrid genes can be cloned into the plasmid which is used to transfect human

30   cell lines to assure recombinant protein expression. The plasmid is propagated in E. coli and purified in quantities sufficient for immunization studies by cesium chloride

gradient centrifugation. Mice are immunized with 50 ug of plasmid in 50 ul saline given intramuscularly into the rectus femoris. Booster injections of the same dose are given at three and six weeks after the initial injection.

5   A wide variety of other gene delivery vehicles can likewise be utilized within the context of the present invention, including for example, viruses, retrotransposons and cosmids. Representative examples include adenoviral vectors (*e.g.*, WO 94/26914, WO 93/9191; Yei et al., *Gene Therapy 1*:192-200, 1994; Kolls et al., *PNAS 91*(1):215-219, 1994; Kass-Eisler et al., *PNAS 90*(24):11498-502, 1993; Guzman et al., *Circulation 88*(6):2838-48, 1993; Guzman et al., *Cir. Res.*

10   *73*(6):1202-1207, 1993; Zabner et al., *Cell 75*(2):207-216, 1993; Li et al., *Hum Gene Ther. 4*(4):403-409, 1993; Caillaud et al., *Eur. J. Neurosci. 5*(10):1287-1291, 1993), adeno-associated type 1 ("AAV-1") or adeno-associated type 2 ("AAV-2") vectors (*see* WO 95/13365; Flotte et al., *PNAS 90*(22):10613-10617, 1993), hepatitis delta vectors, live, attenuated delta viruses and herpes viral vectors (*e.g.*, U.S. Patent No.

15   5,288,641), as well as vectors which are disclosed within U.S. Patent No. 5,166,320. Other representative vectors include retroviral vectors (*e.g.*, EP 0 415 731; WO 90/07936; WO 91/02805; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218). Methods of using such vectors in gene therapy are well known in the art, see, for example, Larrick, J.W. and Burck,

20   K.L., *Gene Therapy: Application of Molecular Biology*, Elsevier Science Publishing Co., Inc., New York, New York, 1991; and Kreigler, M., *Gene Transfer and Expression: A Laboratory Manual*, W.H. Freeman and Company, New York, 1990.

        Gene-delivery vehicles may be introduced into a host cell utilizing a vehicle, or by various physical methods. Representative examples of such methods

25   include transformation using calcium phosphate precipitation (Dubensky et al., *PNAS 81*:7529-7533, 1984), direct microinjection of such nucleic acid molecules into intact target cells (Acsadi et al., *Nature 352*:815-818, 1991), and electroporation whereby cells suspended in a conducting solution are subjected to an intense electric field in order to transiently polarize the membrane, allowing entry of the nucleic acid

30   molecules. Other procedures include the use of nucleic acid molecules linked to an inactive adenovirus (Cotton et al., *PNAS 89*:6094, 1990), lipofection (Felgner et al.,

Proc. Natl. Acad. Sci. USA 84:7413-7417, 1989), microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991), polycation compounds such as polylysine, receptor specific ligands, liposomes entrapping the nucleic acid molecules, spheroplast fusion whereby *E. coli* containing the nucleic acid molecules are stripped of their outer cell walls and fused to animal cells using polyethylene glycol, viral transduction, (Cline et al., *Pharmac. Ther. 29*:69, 1985; and Friedmann et al., *Science 244*:1275, 1989), and DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 1989), as well as psoralen inactivated viruses such as Sendai or Adenovirus.

Serum from mice immunized with gene delivery vehicles containing multivalent M protein genes are assayed for total antibody titer by ELISA using native M proteins as the antigen. Serum opsonic antibodies are assayed as described above. Protective efficacy of DNA M protein vaccines is determined by direct mouse protection tests using the serotypes of group A streptococci represented in the vaccine.

FORMULATION AND ADMINISTRATION

For therapeutic use, vaccinating agents can be administered to a patient by a variety of routes, including for example, by intramuscular, subcutaneous, and mucosal routes. The vaccinating agent may be administered as a single dosage, or in multiple units over an extended period of time. Within preferred embodiments, the vaccinating agent is administered to a human at a concentration of 50-300 ug per single site intramuscular injection. Several injections can be given (*e.g.*, three or four) at least one month apart in order to further increase vaccine efficacy.

Typically, the vaccinating agent will be administered in the form of a pharmaceutical composition comprising purified polypeptide in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to patients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the vaccinating agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose,

sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents.

Within preferred embodiments of the invention, the vaccinating agent is combined with an adjuvant, such as, for example, Freund's adjuvant, alum and the like.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1
CONSTRUCTION AND EXPRESSION OF A HEXAVALENT FUSION GENE

A hexavalent emm gene was constructed using PCR to amplify specific 5' regions of the six different emm genes (24, 5, 6, 19, 1 and 3) essentially as described previously (Dale et al., "Recombinant tetravalent group A streptococcal M protein vaccine," *J. Immunol.* *151*:2188-2194, 1993; Dale et al., "Recombinant, octavalent group A streptococcal M protein vaccine," *Vaccine 14*:944-948, 1996).

Briefly, the multivalent genes are constructed using PCR and primers that specify specific 5' emm gene fragments. The gene fragments may range in size from 30 bp to 300 bp. Chromosomal DNA from each serotype of group A streptococcus is used as the template for the PCR reactions. For the hexavalent emm gene described in the example, the PCR primers are as follows:

M24-1 TS   SphI
5' GGG GGG GCA TCG GTC GCG ACT AGG TCT CAG ACA GAT 3'
(SEQ ID NO:1)

M24-1 BS   BamH1
5' GGG GGG GGA TCC ACG TAG TTT CTC TTT AGC 3'
(SEQ ID NO:2)

M5 TS   BamH1
5' GGG GGG GGA TCC GCC GTG ACT AGG GGT ACA 3'
(SEQ ID NO:3)

M5 BS   SalI
5' GGG GGG GTC GAC CTC AGT TTT TAA CCC TTC 3'
(SEQ ID NO:4)

M6 TS   SalI
5' GGG GGG GTC GAC AGA GTG TTT CCT AGG GGG 3'
(SEQ ID NO:5)

M6 BS   NcoI
5' GGG GGG CCA TGG TAA CTT GTC ATT ATT AGC 3'
(SEQ ID NO:6)

5   M19 TS   NcoI
5' GGG GGG CCA TGG AGA GTG CGT TAT ACT AGG 3'
(SEQ ID NO:7)

M19 BS   PstI
10   5' GGG GGG CTG CAG AGA TAA CTT CTC ATT CTG 3'
(SEQ ID NO:8)

M1 TS   PstI
5' GGG GGG CTG CAG AAC GGT GAT GGT AAT CCT 3'
15   (SEQ ID NO:9)

M1 BS   KpnI
5' GGG GGG GGT ACC AGC TCT CTT AAA ATC TCT 3'
(SEQ ID NO:10)
20

M3 TS   KpnI
5' GGG GGG GGT ACC TTG TTA GAT CAG GTT ACA 3'
(SEQ ID NO:11)

25   M3 BS   ClaI
5' GGG GGG ATC GAT ATT TAA CTC TTG TAA CAG 3'
(SEQ ID NO:12)

M24-2 TS   ClaI
30   5' GGG GGG ATC GAT GTC GCG ACT AGG TCT CAG 3'
(SEQ ID NO:13)

M24-2 BS   HindIII
5' GGG GGG AAG CTT TTA CTT ACG TGC CTC TAA TTC 3'
35   (SEQ ID NO:14)

PCR is performed on the chromosomal template as previously described (Dale et al., "Recombinant tetravalent group A streptococcal M protein vaccine," *J. Immunol.* *151*:2188-2194, 1993). To assure ligation of the fragments in
40   the correct orientation and reading frame, each PCR product is purified, ligated, and then subjected to PCR again using the forward primer from the 5' fragment and the reverse primer from the 3' fragment. For example, to construct a hexavalent emm gene containing DNA sequences from types 24, 5, 6, 19, 1, and 3 M proteins, the

M24 and M5 gene fragments are amplified by PCR using the primers described above. The PCR products are purified from agarose gels, cut with the appropriate restriction enzyme, and ligated together (Dale et al., "Recombinant tetravalent group A streptococcal M protein vaccine," *J. Immunol. 151*:2188-2194, 1993; Dale et al.,

5   "Recombinant, octavalent group A streptococcal M protein vaccine," *Vaccine 14*:944-948, 1996). The ligation mixture is then amplified by PCR using the forward M24 primer and the reverse M5 primer. The resulting product of the appropriate size is then purified and ligated to the M6 and M19 gene fragment that was similarly constructed. After the final ligation reaction, the entire gene is amplified again by

10   PCR, cut with the appropriate restriction enzymes and ligated into a suitable expression vector. For the addition of the reiterated M24 gene fragment in the 3' location, the plasmid was purified from the host E. coli and a new PCR product from emm 24 was force cloned into the 3' PstI restriction site.

The hexavalent gene was sequenced by the dideoxy-nucleotide chain

15   termination method to confirm that each gene fragment was an exact copy of the respective native emm sequence.

EXAMPLE 2

PURIFICATION OF A HEXAVALENT VACCINE

20   A.   Purification

Transformed *E. coli* were grown in a shaking incubator to log phase in 1l of LB containing 100 ug/ml ampicillin and 25 ug/ml kanamycin. IPTG (2mM) was added for the final four hours of growth. The cell pellet was suspended in 30 ml PBS and lysed in a French pressure cell at 1000 psi. The hexavalent protein was

25   purified from the supernatant using Ni-NTA resin according to the protocol provided by the manufacturer (Qiagen). The elution buffer containing the protein was concentrated from 15 ml to 5 ml in a spin filter (Ultrafree-15, Millipore). Final purification was accomplished by gel filtration over Superdex 75 (prep grade, Pharmacia Biotech). The active fraction was identified by Western blots (Dale, J.B.

30   and Beachey, E.H., "Multiple heart-cross-reactive epitopes of streptococcal M

proteins," *J. Exp. Med. 161*:113-122, 1985) using rabbit antiserum against pep M24 (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of the type 24 M antigen," *J. Exp. Med. 145*:1469-1483, 1977). Total protein concentration was

5   determined by standard methods and the sample was diluted in PBS to contain 200 ug/ml of hexavalent protein. Purity of the samples was determined by gel scanning (Photoshop digital image and Collage image analysis).

B.   Analysis of the hexavalent vaccine

The structure of the hybrid emm gene was confirmed by double-

10   stranded sequencing methods after ligation into pQE30. The sequence of each subunit was identical to the respective native emm gene (Figure 1). The fragments were joined only by the two amino acids specified by each unique restriction site used to facilitate their ligation (Figure 1).

The purified hexavalent protein migrated on SDS-polyacrylamide gels

15   with an apparent M.W. of 45 kDa. (Figure 2). Gel scan analysis revealed that the intact hexavalent protein accounted for approximately 90% of the total stainable protein in the gel. Western blots using antisera against pep M24 showed that the majority of the remaining protein bands were immunoreactive and most likely were fragments of the hexavalent protein (data not shown).

20

EXAMPLE 3

IMMUNIZATION OF RABBITS, AND TESTING OF ANTISERA

A.   Immunization

25   Two groups of three rabbits each were immunized with 100 ug of hexavalent vaccine either precipitated with alum or emulsified in complete Freund's adjuvant. For precipitation in alum, the hexavalent protein (200 ug/ml) was added to an equal volume of aluminum hydroxide (2mg/ml) (Rehydragel HPA, Reheis, Inc., Berkeley Heights, NJ) and mixed gently at 40C overnight. The hexavalent protein

was also emulsified in CFA at a final concentration of 100 ug/ml. Rabbits that received the hexavalent vaccine in alum were given 100 ug I.M. as an initial injection and the same dose was repeated at 4, and 8 weeks. The second set of rabbits received 100 ug of hexavalent vaccine in CFA subcutaneously as an initial injection and then

5   booster injections of the same dose in saline were given at 4 and 8 weeks. Blood was obtained prior to the first injection and at 2-week intervals thereafter.

Antibody assays. ELISAs were performed using purified native pepsin-extracted M proteins (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino

10   terminal region of the type 24 M antigen," *J. Exp. Med. 145*:1469-1483, 1977) or the purified hexavalent protein, as previously described (Dale et al., "Heterogeneity of type-specific and cross-reactive antigenic determinants within a single M protein of group A streptococci," *J. Exp. Med. 151*:1026-1038, 1980). Opsonic antibodies were detected by *in vitro* opsonization assays and indirect bactericidal assays (Beachey et

15   al., "Human immune response to immunization with a structurally defined polypeptide fragment of streptococcal M protein," *J. Exp. Med. 150*:862-877, 1979).

B.   Detection of M protein antibodies.

The preimmune and immune animal sera are assayed by ELISA using the vaccine protein and the native pepsin-extracted M proteins as solid-phase

20   antigens (Dale et al., "Heterogeneity of type-specific and cross-reactive antigenic determinants within a single M protein of group A streptococci," *J. Exp. Med. 151*:1026-1038, 1980). ELISA titers are defined as the inverse of the last dilution of antisera resulting in an OD of >0.1 at 450nm. The titers of immune sera against the native M antigen are most likely to predict the levels of antibodies that are evoked by

25   the recombinant protein that will react with the M protein on the surface of the respective serotype of streptococcus (*i.e.* promote opsonization).

C.   Detection of opsonic antibodies.

Opsonic M protein antibodies correlate with protection against infection with the same serotype of group A streptococci (Lancefield, R.C., "Current

knowledge of the type specific M antigens of group A streptococci," *J. Immunol.* *89*:307-313, 1962; Lancefield, R.C., "Persistence of type-specific antibodies in man following infection with group A streptococci," *J. Exp. Med.* *110*:271-282, 1959). Two related in vitro assays are used to detect opsonic antibodies in immune sera.

5   The first is a screening assay that measures opsonization in mixtures of immune serum, whole, nonimmune human blood and the test organism (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of the type 24 M antigen," *J. Exp. Med.* *145*:1469-1483, 1977). 0.1 ml of test serum is added to a standard number

10   of bacteria and incubated for 15 minutes at room temperature. 0.4 ml of lightly heparinized human blood is added and the entire mixture is rotated end-over-end at 370 for 45 minutes. At the end of the rotation, smears are prepared on microscope slides that are air-dried and stained with Wright's stain. "Percent opsonization" is quantitated by counting the percentage of polymorphonuclear leukocytes that have

15   ingested or are associated with bacteria. An interpretable assay must have a preimmune control value that is 10% opsonization or less.

Confirmation of the presence of opsonic antibodies is obtained by indirect bactericidal antibody assays according to the original description by Lancefield (Lancefield, R.C., "Current knowledge of the type specific M antigens of

20   group A streptococci," *J. Immunol.* *89*:307-313, 1962). This assay is performed using test mixtures as described above except that fewer bacteria are added and the rotation is allowed to proceed for 3 hours. At the end of the rotation, pour plates are made in sheep blood agar and bacteria surviving are quantitated after overnight growth at 370. Percent killing in the presence of immune serum is calculated by

25   comparing to the growth in nonimmune serum.

EXAMPLE 4

MOUSE PROTECTION ASSAYS

A.   General Protocol

Protective efficacy of M protein vaccines is determined by either
indirect or direct (passive or active immunization) mouse protection tests. Indirect
tests are performed by giving mice 1 ml of immune or preimmune serum via the
intraperitoneal (i.p.) route 24 hours prior to challenge infections with the test
organism given i.p. (Beachey et al., "Human immune response to immunization with
a structurally defined polypeptide fragment of streptococcal M protein," *J. Exp. Med.*
150:862-877, 1979).   For each test organism, groups of 25 mice receive either
preimmune of immune serum. The animals are then divided into 5 groups of 5 mice
each and 10-fold increasing challenge doses of virulent streptococci are given to each
subgroup. After 7 days of observation, the LD50 is calculated for each serotype
tested.

Direct mouse protection tests are similarly performed except that mice
are actively immunized with M protein vaccine prior to the challenge infections.
Each mouse receives 25-50ug vaccine in alum given intramuscularly (i.m.) at time 0,
4 weeks, and 8 weeks. Challenge infections are performed ten weeks after the first
injection. Control animals are sham immunized with alum alone. The LD50 is
calculated and significance is determined using Fisher's exact test.

B.   Protection

In order to show directly the protective efficacy of opsonic antibodies
evoked by the hexavalent vaccine, mice were immunized with the vaccine adsorbed
to ALUM and then challenged with two of the serotypes represented in the vaccine.
Female outbred white Swiss mice were immunized via the I.M. route in the hind leg
according to the following schedule: time 0, 25ug; 3 weeks, 25 ug; 6 weeks, 50ug;
and 13 weeks, 50ug. Challenge experiments were performed on the 20 immunized
mice and 20 control, unimmunized mice (Table 1.) The challenge strains were types
24 and 19, with the reasoning that the M24 peptide is the largest fragment in the

hexavalent protein and is reiterated and the M19 fragment is one of two that are only 35 amino acids long. These two fragments should reflect the range of protective immunogenicity of the hexavalent protein. Interperitoneal challenge of mice with virulent streptococci is the most stringent laboratory assay for opsonic antibodies.

5   In this experiment, two groups of ten mice each were challenged with an inoculum that approximated the LD_{70}-LD_{100} for each serotype, which was $2x10^4$ CFU. The challenge experiments were begun 15 weeks after the first dose of vaccine was administered and deaths were recorded for 10 days. The mice that were immunized with the hexavalent vaccine and challenged with type 24 streptococci

10 were significantly protected from death compared to the control group (p=.0001). The mice challenged with type 19 streptococci were protected by vaccination, but the level was not statistically significant (p=.15). Had the challenged group been twice the size, the same level of protection would have resulted in a statistically significant survival rate. When the survival of the entire immunized group of mice is analyzed,

15 the level of protection was highly significant (p=.0002).

Table 1. Protective immunogenicity of the hexavalent vaccine in mice that were challenged i.p. with virulent type 24 and type 19 streptococci

Group	#Dead/#Survived of Mice Challenged (% survival)		
	Type 24	Type 19	Total
Immunized mice	0/10 (100)	4/6 (60)	4/16 (80) p=.0002*
Control mice	9/1 (10)	7/3 (30)	16/4 (20)

*p value was calculated using the Fisher exact test.

20   EXAMPLE 5

ASSAYS FOR TISSUE-CROSSREACTIVE ANTIBODIES

       To assure that none of the M protein vaccines evokes tissue-crossreactive antibodies, indirect immunofluorescence assays are performed using

25 frozen sections of human heart, kidney, and brain (Dale, J.B. and Beachey E.H., "Protective antigenic determinant of streptococcal M protein shared with

sarcolemmal membrane protein of human heart," *J. Exp. Med. 156*:1165-1176, 1982). Thin sections of tissue obtained at autopsy (4um) are prepared on microscope slides and stored in a sealed box at -700C until use. Test serum is diluted 1:5 in PBS and dropped onto the tissue section. Control slides are made with preimmune serum and

5   PBS. The slides are incubated at ambient temperature for 30 minutes and then washed three times in PBS in a slide holder. Fluorescein-labeled goat anti-IgG/IgM/IgA is diluted 1:40 in PBS and dropped onto the slides which are again washed, dried, and mounted with 1% Gelvetol and a coverslip. Fluorescence is detected using a Zeiss Axiophot microscope equipped with a xenon light source.

10   Immunofluorescence is recorded using a scale of 0-4+, with 0 being no fluorescence and 4+ being that obtained with a standard, positive antiserum raised in rabbits against whole type 5 M protein (Dale, J.B. and Beachey, E.H., "Multiple heart-cross-reactive epitopes of streptococcal M proteins," *J. Exp. Med. 161*:113-122, 1985).

15

EXAMPLE 6

COMPARISON OF THE IMMUNOGENICITY OF A HEXAVALENT VACCINE
DELIVERED IN ALUM VERSUS FREUND'S ADJUVANT

20   Three rabbits each were immunized with 100 ug doses of the hexavalent vaccine in either alum of CFA. Booster injections of the same dose were given at 4 and 8 weeks in either alum or saline, respectively. ELISA titers were determined using the purified hexavalent protein as the solid phase antigen (Figure 3). Sera from the animals that received the hexavalent vaccine in alum had antibody

25   titers that were equal to or greater than the sera from rabbits that received the same dose in CFA. In a subsequent experiment, three rabbits were immunized I.M. with 100 ug of the hexavalent vaccine in saline alone according the same schedule. None of these rabbits developed significant antibody titers against either the immunogen or the respective pep M proteins (data not shown). These data indicate that alum is a

30   suitable and necessary adjuvant for the multivalent vaccine and is equal to the adjuvant activity of CFA in combination with the hexavalent protein.

EXAMPLE 7

PROTECTIVE IMMUNOGENICITY OF THE COMPONENT SUBUNITS OF A HEXAVALENT VACCINE

5

One of the major goals of this study was to design a multivalent, hybrid protein that retained the immunogenic properties of each M protein subunit. ELISAs were performed on sera obtained from the three rabbits immunized with the hexavalent vaccine in alum (Figure 4). In each case the ELISA antigen was the purified pepsin-extracted M protein. Thus, the assay measures only the antibodies evoked by the hexavalent protein that react with the native M protein and not the antibodies that may be specific for the joining segments or conformations that are not present in the native M proteins. The hexavalent protein evoked significant levels of antibodies against each M protein represented in the vaccine construct (Figure 4). Importantly, none of the antisera contained antibodies that crossreacted with human heart tissue or kidney tissue, as determined by indirect immunofluorescence assays (data not shown).

Sera from all three rabbits contained significant levels of opsonic antibodies against each serotype of group A streptococci represented in the vaccine (Figure 5). These results were confirmed by indirect bactericidal assays using one of the immune sera (Table 2). Taken together, the results indicate that the individual components of the hexavalent vaccine retain the conformation and immunogenicity necessary to elicit antibodies that react with the native M proteins on the surface of each respective serotype of group A streptococci.

25

Table 2. <u>Indirect bactericidal assay of rabbit antiserum against the hexavalent M protein vaccine.</u>

| Serotype | Inoculum(CFU) | CFU Surviving 3 hr rotation: | | Percent |
		Preimmune	Immune	Reduction
24	12	2890	0	100
5	11	3260	0	100
6	6	2640	0	100
19	6	1580	0	100
1	8	2670	490	82
3	11	1720	10	99

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Dale, James B.

    (ii) TITLE OF INVENTION: GROUP A STREPTOCOCCAL VACCINES

    (iii) NUMBER OF SEQUENCES: 16

    (iv) CORRESPONDENCE ADDRESS:
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        (B) STREET: 6300 Columbia Center, 701 Fifth Avenue
        (C) CITY: Seattle
        (D) STATE: Washington
        (E) COUNTRY: USA
        (F) ZIP: 98104

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: US
        (B) FILING DATE: 12-SEP-1997
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
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        (C) REFERENCE/DOCKET NUMBER: 481112.410P1

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (206) 622-4900
        (B) TELEFAX: (206) 682-6031

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 36 base pairs
        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGGGGGGCAT CGGTCGCGAC TAGGTCTCAG ACAGAT   36

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGGGGGGGAT CCACGTAGTT TCTCTTTAGC   30

(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGGGGGGGAT CCGCCGTGAC TAGGGGTACA   30

(2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGGGGGGTCG ACCTCAGTTT TTAACCCTTC   30

(2) INFORMATION FOR SEQ ID NO:5:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGGGGGGTCG ACAGAGTGTT TCCTAGGGGG   30

(2) INFORMATION FOR SEQ ID NO:6:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGGGGCCAT GGTAACTTGT CATTATTAGC   30

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGGGGCCAT GGAGAGTGCG TTATACTAGG   30

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGGGGCTGC AGAGATAACT TCTCATTCTG   30

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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(2) INFORMATION FOR SEQ ID NO:10:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 30 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGGGGGGTA CCAGCTCTCT TAAAATCTCT   30

(2) INFORMATION FOR SEQ ID NO:11:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 30 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGGGGGGGTA CCTTGTTAGA TCAGGTTACA   30

(2) INFORMATION FOR SEQ ID NO:12:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 30 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGGGGGGATCG ATATTTAACT CTTGTAACAG   30

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGGGGGGATCG ATGTCGCGAC TAGGTCTCAG   30

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGGGGGGAAGC TTTTACTTAC GTGCCTCTAA TTC   33

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1158 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:
    (A) NAME/KEY: CDS
    (B) LOCATION: 1..1149

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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GCA TGC ATG GTC GCG ACT AGG TCT CAG ACA GAT ACT CTG GAA AAA GTA          48
Ala Cys Met Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val
 1               5                  10                  15

CAA GAA CGT GCT GAC AAG TTT GAG ATA GAA AAC AAT ACG TTA AAA CTT          96
Gln Glu Arg Ala Asp Lys Phe Glu Ile Glu Asn Asn Thr Leu Lys Leu
            20                  25                  30

AAG AAT AGT GAC TTA AGT TTT AAT AAT AAA GCG TTA AAA GAT CAT AAT         144
Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn
            35                  40                  45

GAT GAG TTA ACT GAA GAG TTG AGT AAT GCT AAA GAG AAA CTA CGT GGA         192
Asp Glu Leu Thr Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Gly
     50                  55                  60

TCC GCC GTG ACT AGG GGT ACA ATA AAT GAC CCG CAA AGA GCA AAA GAA         240
Ser Ala Val Thr Arg Gly Thr Ile Asn Asp Pro Gln Arg Ala Lys Glu
 65                  70                  75                  80

GCT CTT GAC AAG TAT GAG CTA GAA AAC CAT GAC TTA AAA ACT AAG AAT         288
Ala Leu Asp Lys Tyr Glu Leu Glu Asn His Asp Leu Lys Thr Lys Asn
                85                  90                  95

GAA GGG TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG AAT GAA GGG         336
Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Asn Glu Gly
            100                 105                 110

TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG GTC GAC AGA GTG TTT         384
Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Val Asp Arg Val Phe
            115                 120                 125

CCT AGG GGG ACG GTA GAA AAC CCG GAC AAA GCA CGA GAA CTT CTT AAC         432
Pro Arg Gly Thr Val Glu Asn Pro Asp Lys Ala Arg Glu Leu Leu Asn
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        130                    135                    140

AAG TAT GAC GTA GAG AAC TCT ATG TTA CAA GCT AAT AAT GAC AAG TTA        480
Lys Tyr Asp Val Glu Asn Ser Met Leu Gln Ala Asn Asn Asp Lys Leu
145                 150                 155                 160

CCA TGG AGA GTG CGT TAT ACT AGG CAT ACG CCA GAA GAT AAG CTA AAA        528
Pro Trp Arg Val Arg Tyr Thr Arg His Thr Pro Glu Asp Lys Leu Lys
                165                 170                 175

AAA ATT ATT GAC GAT CTT GAC GCA AAA GAA CAT GAA TTA CAA CAA CAG        576
Lys Ile Ile Asp Asp Leu Asp Ala Lys Glu His Glu Leu Gln Gln Gln
                180                 185                 190

AAT GAG AAG TTA TCT CTG CAG AAC GGT GAT GGT AAT CCT AGG GAA GTT        624
Asn Glu Lys Leu Ser Leu Gln Asn Gly Asp Gly Asn Pro Arg Glu Val
            195                 200                 205

ATA GAA GAT CTT GCA GCA AAC AAT CCC GCA ATA CAA AAT ATA CGT TTA        672
Ile Glu Asp Leu Ala Ala Asn Asn Pro Ala Ile Gln Asn Ile Arg Leu
        210                 215                 220

CGT CAC GAA AAC AAG GAC TTA AAA GCG AGA TTA GAG AAT GCA ATG GAA        720
Arg His Glu Asn Lys Asp Leu Lys Ala Arg Leu Glu Asn Ala Met Glu
225                 230                 235                 240

GTT GCA GGA AGA GAT TTT AAG AGA GCT GGT ACC TTG TTA GAT CAG GTT        768
Val Ala Gly Arg Asp Phe Lys Arg Ala Gly Thr Leu Leu Asp Gln Val
                245                 250                 255

ACA CAA TTA TAT ACT AAA CAT AAT AGT AAT TAC CAA CAA TAT AAT GCA        816
Thr Gln Leu Tyr Thr Lys His Asn Ser Asn Tyr Gln Gln Tyr Asn Ala
                260                 265                 270

CAA GCT GGC AGA CTT GAC CTG AGA CAA AAG GCT GAA TAT CTA AAA GGC        864
Gln Ala Gly Arg Leu Asp Leu Arg Gln Lys Ala Glu Tyr Leu Lys Gly
            275                 280                 285

CTT AAT GAT TGG GCT GAG AGG CTG TTA CAA GAG TTA AAT ATC GAT GTC        912
Leu Asn Asp Trp Ala Glu Arg Leu Leu Gln Glu Leu Asn Ile Asp Val
        290                 295                 300

GCG ACT AGG TCT CAG ACA GAT ACT CTG GAA AAA GTA CAA GAA CGT GCT        960
Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala
305                 310                 315                 320
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GAC AAG TTT GAG ATA GAA AAC AAT ACG TTA AAA CTT AAG AAT AGT GAC          1008
Asp Lys Phe Glu Ile Glu Asn Asn Thr Leu Lys Leu Lys Asn Ser Asp
            325                 330                 335


TTA AGT TTT AAT AAT AAA GCG TTA AAA GAT CAT AAT GAT GAG TTA ACT          1056
Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn Asp Glu Leu Thr
            340                 345                 350


GAA GAG TTG AGT AAT GCT AAA GAG AAA CTA CGT AAA AAT GAT AAA TCA          1104
Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Lys Asn Asp Lys Ser
            355                 360                 365


CTA TCT GAA AAA GCT AGT AAA ATT CAA GAA TTA GAG GCA CGT AAG              1149
Leu Ser Glu Lys Ala Ser Lys Ile Gln Glu Leu Glu Ala Arg Lys
    370                 375                 380


TAAAAGCTT                                                                 1158
```

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 383 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Ala Cys Met Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val
 1               5                  10                  15


Gln Glu Arg Ala Asp Lys Phe Glu Ile Glu Asn Asn Thr Leu Lys Leu
            20                  25                  30


Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn
            35                  40                  45


Asp Glu Leu Thr Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Gly
    50                  55                  60


Ser Ala Val Thr Arg Gly Thr Ile Asn Asp Pro Gln Arg Ala Lys Glu
 65                  70                  75                  80
```

Ala Leu Asp Lys Tyr Glu Leu Glu Asn His Asp Leu Lys Thr Lys Asn
                85   90   95

Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Asn Glu Gly
            100   105   110

Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Val Asp Arg Val Phe
            115   120   125

Pro Arg Gly Thr Val Glu Asn Pro Asp Lys Ala Arg Glu Leu Leu Asn
        130   135   140

Lys Tyr Asp Val Glu Asn Ser Met Leu Gln Ala Asn Asn Asp Lys Leu
145   150   155   160

Pro Trp Arg Val Arg Tyr Thr Arg His Thr Pro Glu Asp Lys Leu Lys
                165   170   175

Lys Ile Ile Asp Asp Leu Asp Ala Lys Glu His Glu Leu Gln Gln Gln
                180   185   190

Asn Glu Lys Leu Ser Leu Gln Asn Gly Asp Gly Asn Pro Arg Glu Val
            195   200   205

Ile Glu Asp Leu Ala Ala Asn Asn Pro Ala Ile Gln Asn Ile Arg Leu
        210   215   220

Arg His Glu Asn Lys Asp Leu Lys Ala Arg Leu Glu Asn Ala Met Glu
225   230   235   240

Val Ala Gly Arg Asp Phe Lys Arg Ala Gly Thr Leu Leu Asp Gln Val
                245   250   255

Thr Gln Leu Tyr Thr Lys His Asn Ser Asn Tyr Gln Gln Tyr Asn Ala
            260   265   270

Gln Ala Gly Arg Leu Asp Leu Arg Gln Lys Ala Glu Tyr Leu Lys Gly
        275   280   285

Leu Asn Asp Trp Ala Glu Arg Leu Leu Gln Glu Leu Asn Ile Asp Val
        290   295   300

Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala
305   310   315   320

Asp Lys Phe Glu Ile Glu Asn Asn Thr Leu Lys Leu Lys Asn Ser Asp
                325   330   335

Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn Asp Glu Leu Thr
                340   345   350

Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Lys Asn Asp Lys Ser
            355   360   365

Leu Ser Glu Lys Ala Ser Lys Ile Gln Glu Leu Glu Ala Arg Lys
    370   375   380

CLAIMS

I claim:

1.   An immunogenic fusion polypeptide which stimulates an immune response against group A streptococci, comprising:

    (a)   at least two immunogenic polypetides from a group A streptococci of at least 10 amino acids in length which are capable of stimulating an immune response against group A strepococci; and

    (b)   a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic portion, wherein the C-terminal peptide is not required to stimulate an immune response against group A streptococci.

2.   The polypeptide according to claim 1 wherein one of said immunogenic polypeptides is obtained from a serotype 5 group A streptococci.

3.   The polypeptide according to claim 1 wherein one of said immunogenic polypeptides is obtained from a serotype 6 group A streptococci.

4.   The polypeptide according to claim 1 wherein one of said immunogenic polypeptides is obtained from a group A streptococci serotype selected from the group consisting of 1, 1.1, 2, 3, 4, 11, 12, 13, 14, 18, 19, 22, 24, 28, 30, 48, 49, 52 and 56.

5.   A vaccinating agent for promoting an immune response against group A streptococci, comprising:

    (a)   at least two immunogenic polypetides from a group A streptococci of at least 10 amino acids in length which are capable of stimulating a protective immune response against group A strepococci; and

(b)   a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic portion, wherein the C-terminal peptide is not required to stimulate an immune response against group A streptococci.

6.   The vaccinating agent according to claim 5 wherein one of said immunogenic polypeptides is obtained from a serotype 5 group A streptococci.

7.   The vaccinating agent according to claim 5 wherein one of said immunogenic polypeptides is obtained from a serotype 6 group A streptococci.

8.   The vaccinating agent according to claim 5 wherein one of said immunogenic polypeptides is obtained from a group A streptococci serotype selected from the group consisting of 1, 1.1, 2, 3, 4, 11, 12, 13, 14, 18, 19, 22, 24, 28, 30, 48, 49, 52 and 56.

9.   The vaccinating agent according to claim 5, further comprising an adjuvant.

10.   The vaccinating agent according to claim 9 wherein the adjuvant is alum or Freund's adjuvant.

11.   A method for vaccinating a host against group A streptococci infections, comprising administering a vaccinating agent according to any one of claims 5 to 7.

GROUP A STREPTOCOCCAL VACCINES

ABSTRACT OF THE DISCLOSURE

The present invention provides immunogenic synthetic fusion polypeptide which stimulates an immune response against a selected pathogen, comprising at least two immunogenic polypetides from a Group A streptococci of at least 10 amino acids in length which are capable of stimulating an immune response against Group A strepococci, and a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic portion, wherein the C-terminal peptide is not required to stimulate an immune response against Group A streptococci.

\WPN\481112\410P1-AP-2\V1

FIGURE 1

SphI	BamHI	SalI	NcoI	PstI	KpnI	ClaI	HindIII	
5' M24	M5	M6	M19	M1.0	M3		M24	3'
60 Codons	58 Codons	35 Codons ea.	50 Codons	50 Codons		80 Codons		

FIGURE 2

% of the main band:
89 89 86.   89 87 87

FIGURE 3

A

ALUM

(Y-axis: ELISA TITER, values from bottom to top: <100, 200, 400, 800, 1,600, 3,200, 6,400, 12,800, 25,600, 51,200, 102,400, 204,800)

(X-axis: WEEKS, 0 2 4 6 8 10)

B

CFA

(Y-axis: ELISA TITER, values from bottom to top: <100, 200, 400, 800, 1,600, 3,200, 6,400, 12,800, 25,600, 51,200, 102,400, 204,800)

(X-axis: WEEKS, 0 2 4 6 8 10)

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7A

HEXAVALENT M PROTEIN VACCINE SEQUENCE

```
          10          20          30          40          50          60          70
    *      *    *      *    *      *    *      *    *      *    *      *    *      *    *
GCA TGC ATG GTC GCG ACT AGG TCT CAG ACA GAT ACT CTG GAA AAA GTA CAA GAA CGT CCT GAC AAG TTT GAG ATA
Ala Cys Met Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala Asp Lys Phe Glu Ile
-SphI-  M24----->

    80          90          100         110         120         130         140         150
    *      *    *      *    *      *    *      *    *      *    *      *    *      *    *      *
GAA AAC AAT ACG TTA AAA CTT AAG AAT AGT GAC TTA AGT TTT AAT AAT AAA GCG TTA AAA GAT CAT AAT GAT GAG
Glu Asn Asn Thr Leu Lys Leu Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn Asp Glu

          160         170         180         190         200         210         220
    *      *    *      *    *      *    *      *    *      *    *      *    *      *    *
TTA ACT GAA GAG TTG AGT AAT GCT AAA GAG AAA CTA CGT GGA TCC GCC GTG ACT AGG CGT ACA ATA AAT GAC CCG
Leu Thr Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Gly Ser Ala Val Thr Arg Gly Thr Ile Asn Asp Pro
                                               -BamHI- M5------->

    230         240         250         260         270         280         290         300
    *      *    *      *    *      *    *      *    *      *    *      *    *      *    *      *
CAA AGA GCA AAA GAA GCT CTT GAC AAG TAT GAG CTA GAA AAC CAT GAC TTA AAA ACT AAG AAT GAA GGG TTA AAA
Gln Arg Ala Lys Glu Ala Leu Asp Lys Tyr Glu Leu Glu Asn His Asp Leu Lys Thr Lys Asn Glu Gly Leu Lys

          310         320         330         340         350         360         370
    *      *    *      *    *      *    *      *    *      *    *      *    *      *    *
ACT GAG AAT GAA GGG TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG GTC GAC
Thr Glu Asn Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Val Asp
                                                                                         -SalI-

    380         390         400         410         420         430         440         450
    *      *    *      *    *      *    *      *    *      *    *      *    *      *    *      *
AGA GTG TTT CCT AGG GGG ACG GTA GAA AAC CCG GAC AAA GCA CCA GAA CTT CTT AAC AAG TAT GAC GTA GAG AAC
Arg Val Phe Pro Arg Gly Thr Val Glu Asn Pro Asp Lys Ala Arg Glu Leu Leu Asn Lys Tyr Asp Val Glu Asn
M6------->

          460         470         480         490         500         510         520
    *      *    *      *    *      *    *      *    *      *    *      *    *      *    *
TCT ATG TTA CAA GCT AAT AAT GAC AAG TTA CCA TGG AGA GTG CGT TAT ACT AGG CAT ACG CCA GAA GAT AAG CTA
Ser Met Leu Gln Ala Asn Asn Asp Lys Leu Pro Trp Arg Val Arg Tyr Thr Arg His Thr Pro Glu Asp Lys Leu
                               -NcoI- M19----->

    530         540         550         560         570         580         590         600
    *      *    *      *    *      *    *      *    *      *    *      *    *      *    *      *
AAA AAA ATT ATT GAC GAT CTT GAC GCA AAA GAA CAT GAA TTA CAA CAA CAG AAT GAG AAG TTA TCT CTG CAG AAC
Lys Lys Ile Ile Asp Asp Leu Asp Ala-Lys Glu His Glu Leu Gln Gln Gln Asn Glu Lys Leu Ser Leu Gln Asn
                                                                                   -PstI- M1.0

          610         620         630         640         650         660         670
    *      *    *      *    *      *    *      *    *      *    *      *    *      *    *
GGT GAT GGT AAT CCT AGG GAA GTT ATA GAA GAT CTT GCA GCA AAC AAT CCC GCA ATA CAA AAT ATA CGT TTA CGT
Gly Asp Gly Asn Pro Arg Glu Val Ile Glu Asp Leu Ala Ala Asn Asn Pro Ala Ile Gln Asn Ile Arg Leu Arg
------->
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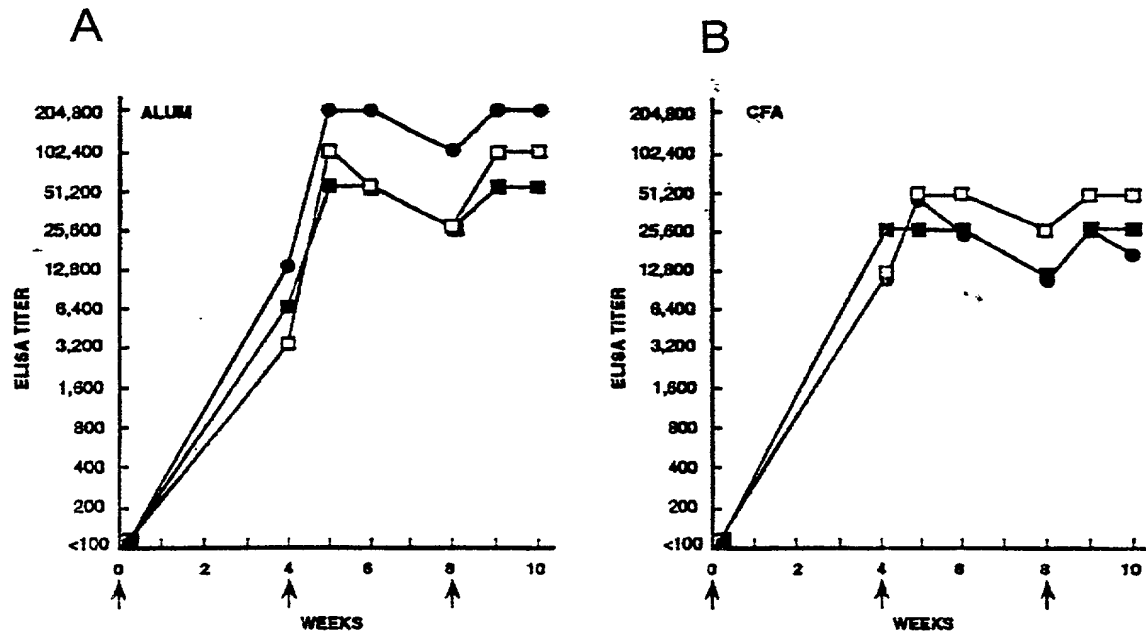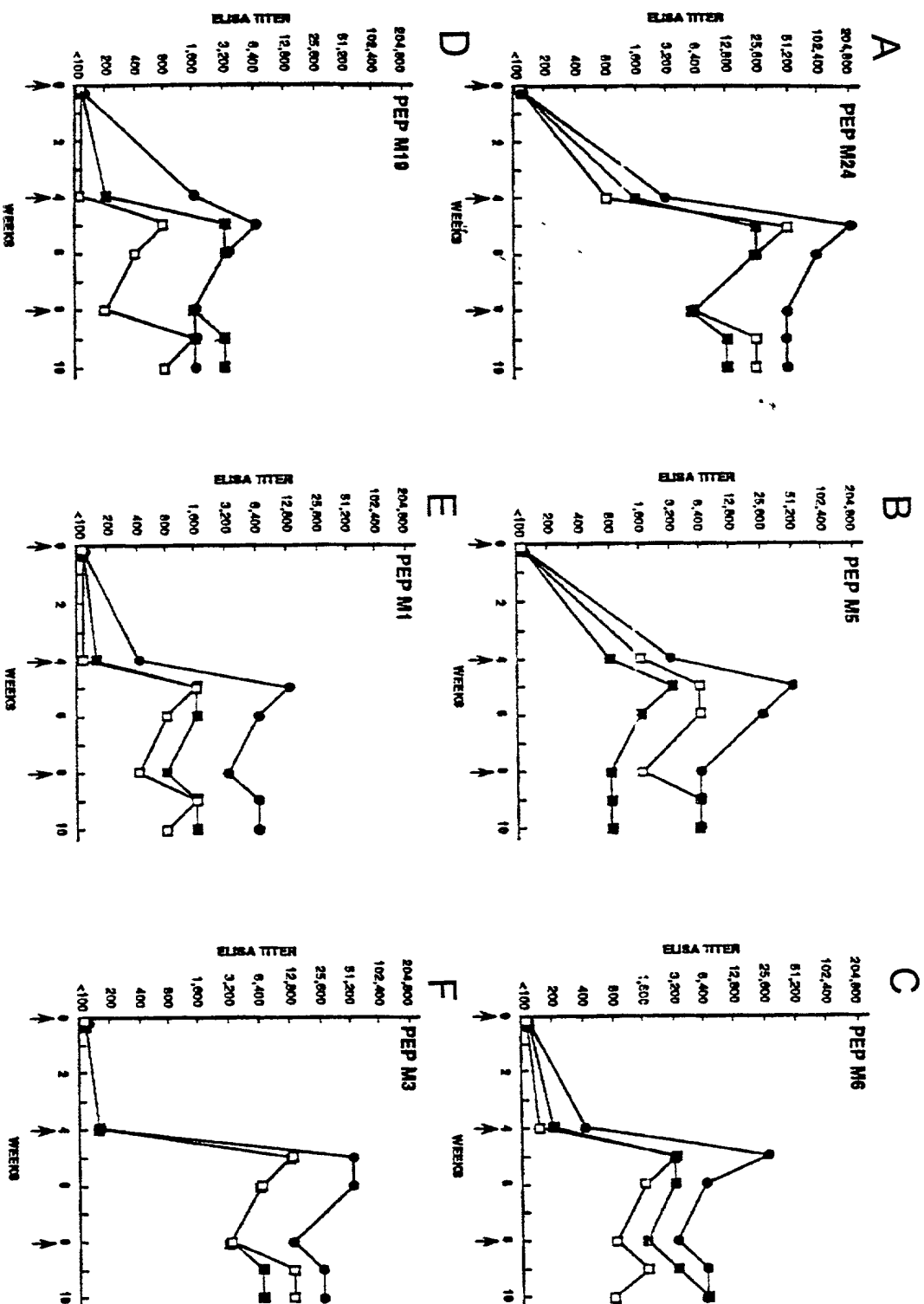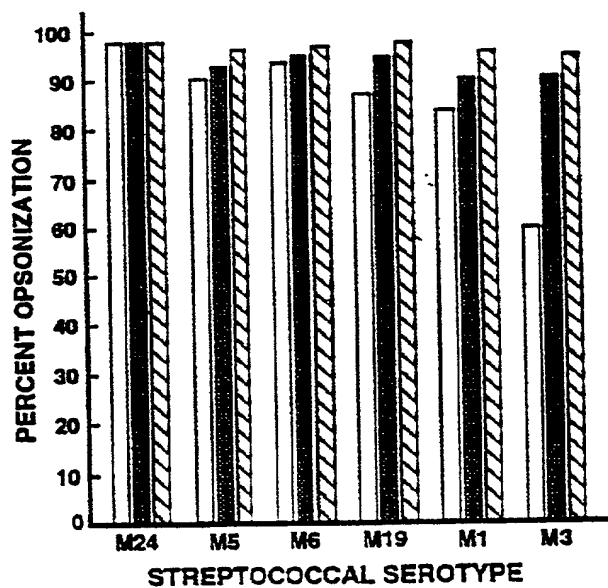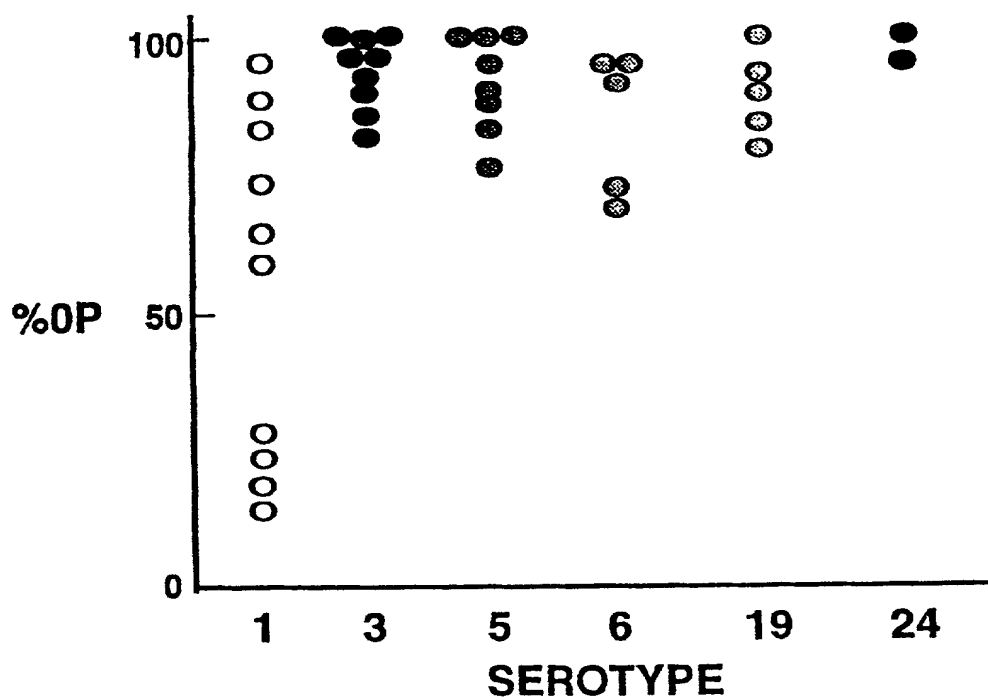
FIGURE 7B

```
         680           690           700          '710          720           730           740         750
    *      *     *      *      *     *      *      *      *      *      *     *      *      *     *
CAC GAA AAC AAG GAC TTA AAA GCG AGA TTA GAG AAT GCA ATG GAA GTT GCA GGA AGA CAT TTT AAG AGA GCT GGT
His Glu Asn Lys Asp Leu Lys Ala Arg Leu Glu Asn Ala Met Glu Val Ala Gly Arg Asp Phe Lys Arg Ala Gly
                                                                                                    KpnI

            760           770           780          790          800           810           820
    *      *     *      *      *     *      *      *     `  *      *     *      *      *      *     *
ACC TTG TTA GAT CAG GTT ACA CAA TTA TAT ACT AAA CAT AAT AGT AAT TAC CAA CAA TAT AAT GCA CAA GCT GGC
Thr Leu Leu Asp Gln Val Thr Gln Leu Tyr Thr Lys His Asn Ser Asn Tyr Gln Gln Tyr Asn Ala Gln Ala Gly
--- M3----->

         830           840           850          860          870           880           890         900
    *      *     *      *      *     *      *      *      *      *      *     *      *      *     *
AGA CTT GAC CTG AGA CAA AAG GCT GAA TAT CTA AAA GGC CTT AAT GAT TGG GCT GAG AGG CTG TTA CAA GAG TTA
Arg Leu Asp Leu Arg Gln Lys Ala Glu Tyr Leu Lys Gly Leu Asn Asp Trp Ala Glu Arg Leu Leu Gln Glu Leu

            910           920           930          940          950           960          970
    *      *     *      *      *     *      *      *      *      *      *     *      *      *     *
AAT ATC GAT GTC GCG ACT AGG TCT CAG ACA GAT ACT CTG GAA AAA GTA CAA GAA CGT GCT GAC AAG TTT GAG ATA
Asn Ile Asp Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala Asp Lys Phe Glu Ile
           -ClaI-  M24----->

         980           990          1000          1010         1020          1030          1040        1050
    *      *     *      *      *     *      *      *      *      *      *     *      *      *     *
GAA AAC AAT ACG TTA AAA CTT AAG AAT AGT GAC TTA AGT TTT AAT AAT AAA GCG TTA AAA GAT CAT AAT GAT GAG
Glu Asn Asn Thr Leu Lys Leu Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn Asp Glu

            1060          1070          1080         1090         1100          1110          1120
    *      *     *      *      *     *      *      *      *      *      *     *      *      *     *
TTA ACT GAA GAG TTG AGT AAT GCT AAA GAG AAA CTA CGT AAA AAT GAT AAA TCA CTA TCT GAA AAA GCT AGT AAA
Leu Thr Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Lys Asn Asp Lys Ser Leu Ser Glu Lys Ala Ser Lys

    1130          1140          1150
    *      *     *      *      *     *
ATT CAA GAA TTA GAG GCA CGT AAG TAA AAG CTT
Ile Gln Glu Leu Glu Ala Arg Lys *** Lys Leu
                              Stop HindIII
```